State of Wisconsin \ DEPARTMENT OF NATURAL RESOURCES

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To all certified and registered WET labs

Subject: Modifications to the fathead minnow chronic test

In most whole effluent toxicity (WET) tests required for compliance in Wisconsin, receiving water is required to be used as the dilution water in WET tests, in order to simulate what happens when the effluent is introduced into the environment. Since Wisconsin's WET program began in the late 80's, an abnormally high failure rate (often $\geq$ 25%) has been noted with chronic fathead minnow tests where receiving water was used as the diluent. In these tests, lowered survival in receiving water controls is noted, there's high variability among replicates in effluent treatments and receiving water controls, and the presence of fungal growth on the gills of dying fish is often noted. All of these factors suggest that a "pathogen effect", or biological interference, is responsible for these unacceptable tests. The pathogen effect almost always confounds test results and makes data unusable (i.e., one cannot always determine whether effluent toxicity was present at critical concentrations). The pathogen effect is not unique to Wisconsin and has been noted by other states and researchers using the same or similar test methods (WET methods originally developed by USEPA).

At the request of the Department, research was done at the UW-Madison State Lab of Hygiene (SLH) to determine the cause of the pathogen effect. The SLH successfully argued that these effects were due to a pathogenic interference and not due to receiving water or effluent toxicity. As a result of the SLH research, minor modifications to the current WET test method can be made which eliminates the pathogen effect. According to the "State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, Edition 1", which is currently referenced in chs. NR 149.22 and 219.04, Wis. Adm. Code, 4 replicates (400 ml each) with 10 fish in each replicate is required in the chronic fathead minnow test (as you know, this is also the test setup required by current USEPA methods in 40 CFR, Part 136). The SLH found that 10 replicates, using smaller test chambers (30 ml), with 2 fish per replicate was successful in eliminating the pathogen effect. The SLH plans to submit these research results to a peer-reviewed, scientific journal for publication sometime in 2002.

Because this small modification to the existing chronic fathead minnow method is successful at removing the pathogen effect from the test without modifying the potential toxicity of effluent or receiving water samples, the Department is pursuing rule revisions to modify the Methods Manual which will require that all chronic fathead minnow tests be conducted using these methods. The Department's WET Methods Manual Team is proposing a modification to chronic fathead minnow test methods in the 2nd edition of the Methods Manual (to include the use of smaller test chambers with 2 fish per chamber). Revisions to chs. NR 149 and 219, Wis. Adm. Code, which would make the 2nd edition of the Methods Manual effective, are expected to be completed sometime in 2003 or 2004. The USEPA is aware of and has acknowledged the results of the SLH study. They've referred to the SLH study and the proposed methods modification in their "Proposed Changes to Whole Effluent Toxicity Manuals" (September 2001) and have indicated that these changes to the fathead minnow chronic test methods will appear in USEPA methods when final revisions are made sometime in the next year or so.

The Department's WET Team has included a number of external participants in the development of drafts of the 2nd edition of the Methods Manual, including WET lab and WPDES permittee representatives, through a series of external meetings. The participants in these external meetings have been very supportive of these proposed changes to the fathead minnow methods and have voiced support regarding the use of them in the interim until rule revisions requiring the 2nd edition of the Methods Manual have been made. Comments regarding proposed changes to the Methods Manual were collected in written form and at external "workshops" in September 2001 and only positive comments were received regarding the proposed changes to the fathead minnow methods.

Language in the current Methods Manual (Edition 1) allows for modifications to test methods if necessary for the successful completion of the test. Section 3.9.3 states that *"Other needs or circumstances may justify modification of or substitution to the toxicity test procedures. Deviation from standard procedures, if necessary for the successful completion of the test battery, may be allowed if first approved by the Department."* It is the opinion of the Department that these modifications to the fathead minnow chronic test method (10 replicates, 30 ml test chambers, 2 fish per replicate) are necessary for the successful completion of the test. With this letter, the Department is asking your lab to begin using this modified version of the fathead minnow chronic method (see attached method) as soon as possible, in place of the method given in Edition 1 of the Methods Manual. Tests conducted using this new method will be accepted by the Department and used for determining WPDES permit compliance.

Questions or comments regarding the attached method can be directed to Steve Geis at the SLH (608-224-6230 or sgeis@mail.slh.wisc.edu) or to me (608-267-7663 or flemik@dnr.state.wi.us). Thanks!

Sincerely,

Kari Fleming
Biomonitoring Coordinator
Bureau of Watershed Management

Cc: Diane Drinkman - IS/6
Bob Weber - WT/2
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From proposed "State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, Edition 2"– February 2002

4.21.4 FATHEAD MINNOW (*PIMEPHALES PROMELAS*) SUB-CHRONIC STATIC RENEWAL PROCEDURES

4.21.4.1 SUMMARY OF METHOD

4.21.4.1.1 Fathead minnow larvae (<24 hours old) are exposed in a static renewal system for 7 days to different concentrations of effluent and receiving water. Results are based on survival and growth. For guidance on fathead minnow culturing methods, refer to pp. 59-68 of "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (USEPA, 1994).

4.21.4.2 START OF THE TEST

4.21.4.2.1 Test solutions must be block randomized using a template or a table of random numbers. When using the randomized block design, test chambers are randomized only once, at the beginning of the test. A number of different templates should be prepared, so that the same template is not used for every test. The larvae should then be pooled and placed into each 30 mL test chamber until each chamber contains 2 larvae for a total of 20 larvae for each concentration. The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

4.21.4.3 FEEDING

4.21.4.3.1 The fish in each test chamber should be fed 0.1 ml of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or 0.15 ml should be fed twice daily at an interval of 6 h. Larvae are not fed during the final 12-h of the test. *(NOTE: amounts of food added to cups may need to be adjusted to account for new method using smaller test chambers - the SLH has found that the previous feeding rate divided by 5 works well).*

4.21.4.3.2 The nauplii should be rinsed with freshwater before use. The amount of food provided should be sufficient to ensure the presence of a small amount of uneaten food at the next feeding.

4.21.4.4 -DAILY RENEWAL

4.21.4.4.1 At the time of daily renewal the fish are transferred to a new test chamber containing fresh test solution using a plastic Pasteur pipet, which has been trimmed at the end to create a 5mm bore diameter. Water transfer is kept to a minimum by allowing the fish to swim out of the pipet into the new test chamber. Injuries to individual fish should be noted on the test sheets.

4.21.4.5 OBSERVATIONS DURING THE TEST

4.21.4.5.1 The number of live and dead larvae in each chamber are recorded daily and the dead discarded.

4.21.4.5.2 To protect the larvae from unnecessary disturbance during the test, daily test observations, and fish transfer should be done carefully. The larvae should remain immersed during the performance of the above operations.

4.21.4.5.3 Animals should be carefully observed during the test for abnormal behavior, such as uncoordinated swimming. Although developmental and behavioral effects are often difficult to quantify and may not provide suitable endpoints, they might be useful for interpreting effects on survival, growth, and reproduction. Morphological examination of organisms alive at the end of the test might be useful as well. WET Test Report Forms should include documentation of any observed abnormal behavior.

4.21.4.6 TERMINATION OF THE TEST

4.21.4.6.1 The test shall be terminated after 7 days of exposure. At termination, the surviving larvae in each chamber should be counted and recorded. For dry weight analysis, replicates are combined in pairs using a random number table, resulting in 5 replicates for weight analysis. Immediately prior to the dry weight analysis, each group should be anaesthetized and dipped in distilled water to remove food particles. Anaesthetized fish are then transferred to a tared weighing boat, and dried at 100°C for a minimum of 2 hours. Immediately upon removal from the drying oven, the weighing boats must be placed in a desiccator until weighed, to prevent the absorption of moisture from the air. All weights should be measured to the nearest 0.01 mg. If the larvae are preserved, they must be dried and weighed within 2 weeks. Preservation is not recommended, but if necessary, must be achieved by freezing.

TABLE 4.4 CHRONIC TEST CONDITIONS FOR FATHEAD MINNOW STATIC RENEWAL TESTS

1. Test type:	Static renewal
2. Test duration:	7 days
3. Temperature (°C):	25 $\pm$ 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50-100 ft-c (540-1075 lux)
6. Photoperiod:	16-h light, 8-h darkness
7. Test chamber size:	30 ml
8. Test solution volume:	20 ml/replicate
9. Renewal of test concentrations:	Daily
10. Age of test organisms:	< 24-h old
11. No. organisms per test chamber:	2
12. No. replicate chambers per concentration:	~~4~~ Minimum 10
13. No. organisms per concentration:	Minimum 20
14. Feeding regime:	Feed 0.1 ml < 24-h brine shrimp nauplii 3x daily at 4-h intervals or 0.15 ml 2x daily, at 6-h intervals. Larvae are not fed during the final 12-h of the test; No additives allowed to food
15. Cleaning:	Transfer fish to new chambers daily.
16. Aeration:	None, unless DO $\leq$ 40% saturation. Rate should not exceed 100 bubbles/min.
17. Dilution water:	Receiving water or synthetic water (see Section 4.3)
18. Dilution series:	If IWC 1-30%, then - 100, 30, 10, 3, 1%; if IWC 31-100%, then - 100, 75, 50, 25, 12.5%; + any additional selected by permittee or alternate dilutions specified in the WPDES permit (see 4.1.2)
19. Test acceptability:	One control must have Survival $\geq$ 80% ,CV between replicates $\leq$ 40%, and avg. dry weight $\geq$ 0.25 mg
20. Sampling requirement:	Specified in WPDES permit and Section 2